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(57) Abstract

The invention provides a method for diagnosis or prediction of relapse or monitoring of disease activity in a patient with neurological disease, particularly multiple sclerosis, comprising quantifying the amount of MMP-9, fragments thereof or allelic variants thereof, and/or a tissue inhibitor of metalloproteinase (TIMP), fragments thereof or allelic variants thereof, present in the blood of a patient, and comparing that amount with a control.

DIAGNOSIS AND MONITORING OF NEUROLOGICAL DISEASE

Field of the invention

This invention relates to methods for the diagnosis and monitoring of the disease process in multiple sclerosis (MS) and other neurological diseases of the central and peripheral nervous system.

Background to the invention

Multiple sclerosis (MS) is an inflammatory disease of the brain and spinal cord characterised by the formation of focal lesions (plaques) in which demyelination and cell death occurs. Within acute lesions the blood brain barrier is damaged, there is an accumulation of activated lymphocytes and macrophages, and resident glial cells such as microglia and astrocytes become activated. The infiltrating leukocytes, together with the activated glial cells, destroy the myelin sheath which surrounds and insulates the nerve axons. More extensive, permanent damage occurs in chronic lesions in which there is destruction of oligodendrocytes and neurons and the formation of a glial cell scar. The demyelination and cell death disrupts nerve conduction, leading to the functional deficits seen in mutiple sclerosis and the other demyelinating diseases.

There is currently no known cure for multiple sclerosis and available treatments have limited benefit. Current therapy includes the use of drugs for the treatment of psychological symptoms, muscle spasm and management of bowel and bladder disfunction. Exacerbations of the disease are controlled by immunosuppressive drugs, with high dose steroids being the most effective at reducing symptoms. However, this treatment requires hospitalisation of the patient and is associated with serious side effects. Although steroids are useful for reducing the symptoms of an exacerbation of the disease, they appear to have no effect on disease progression. A recently introduced immunomodulatory treatment using recombinant interferon beta shows benefit in the reduction of relapse rate but it has to be administered by intramuscular injection twice a

week, and is not always well tolerated. In addition, only a subset of patients with MS are suitable for the treatment and many patients develop neutralising antibodies to the drug. Due to the adverse effects of the available treatments accurate clinical assessment of the patients is of utmost importance, as this will enable the clinician to determine precisely when to administer or withdraw treatment.

Diagnosis of multiple sclerosis is made on clinical assessment of the patients' disabilities, the presence of oligoclonal antibody bands by isoelectric focusing of cerebrospinal fluid (CSF), or the appearance of lesions in the brain and spinal cord in magnetic resonance imaging (MRI) scans. Unfortunately, the presence of oligoclonal bands in the CSF and the lesion load detected by MRI scanning do not correlate with the degree of disability of the patient and are not predictive of an exacerbation of the disease. The appearance of new lesions detected by MRI scanning is indicative of disease progression, but the neurological damage is often subclinical, having no effect on the disability status of the patient.

It has been desirable to provide relatively inexpensive, simple diagnostic and monitoring methods for neurological diseases such as multiple sclerosis which can select patients most likely to respond to available treatments and reduce unnecessary administration of potent drugs such as the steroids. WO 97/09038 (University of New Mexico), is directed to the diagnosis and monitoring of neurological diseases and disorders such as multiple sclerosis, by detection of gelatinase B in cerebrospinal fluid of patients using zymographic techniques. They observed elevated levels of gelatinase B in the cerebrospinal fluid of MS patients, particularly in those with enhancement on the MRI. Following steroid treatment (high dose methylprednisolone), there was a drop in gelatinase B levels in the CSF.

Analysis of blood for a marker of disease activity would provide a practical and economical means for monitoring patients, requiring little specialist expertise and allowing frequent sampling. This is not the case for CSF analysis, as collection of CSF cannot be undertaken as frequently as blood sampling and specialist expertise is

required. CSF collection is increasingly being superseded by MRI scanning in the diagnosis of MS. A blood test for monitoring disease activity would also be an advantage over MRI scanning as lesion load detected does not correlate with clinical relapse and is expensive to carry out. At present there are, however, no blood tests available which give a measure of disease severity or predict or indicate an exacerbation of the disease.

The matrix metalloproteinases (MMPs) are a family of endopeptidases which share a common zinc-containing catalytic domain and are expressed as inactive precursors (Birkedal-Hansen et al (1993) Critical Reviews in Oral Biology and Medicine 4:197-250). A wide range of cells and tissues can express MMPs in response to activation by inflammatory stimuli such as interleukin-1 or tumour necrosis factor alpha (TNF). Different stimuli can induce overlapping, yet distinctive, repertoires of MMPs and different cell types can respond to the same stimuli by expression of distinct combinations of MMPs. Cellular production of MMPs is a tightly controlled process, involving regulation at the level of expression and activation of the pro-enzyme. MMPs can degrade the protein components of the extracellular matrix such as collagens, fibronectin, vitronectin, aggrecan and elastin, and have recently been shown to process membrane proteins including the precursor form of TNF to release the mature form of the cytokine. Inappropriate regulation of MMP activity is thought to play a central role in the pathology of inflammatory diseases such as rheumatoid arthritis and demyelinating neuropathies, such as MS, and in the growth and metastasis of tumours.

The natural inhibitors of metalloproteinases are termed TIMPs (tissue inhibitors of metalloproteinases) and are often found in complex association with metalloproteinases.

MMP-9, also known as 92kDa gelatinase or gelatinase B, has been implicated in the pathology of demyelinating diseases such as MS (Opdenakker et al. (1994) Immunol Today. 15:103-107. MMP-9 can modulate the passage of leukocytes through the extracellular matrix. It has also been demonstrated that MMP-9 can degrade myelin basic protein, a major protein of the myelin sheath, producing immunogenic fragments.

TNF, a potent pro-inflammatory cytokine also implicated in the pathogenesis of MS, has been shown to be processed by MMPs such as MMP-9 (Gearing AJH et al. (1994) Nature. 370:555-557). In an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), MMP-9 is elevated in the CSF of animals developing the disease and inhibitors of MMP or TNF activity reduce symptoms associated with the disease (Gijbels K et al. (1994) J Clin Invest. 94:2177-2182, and Selmaj K et al. (1991) Ann Neurol. 30:694-700).

Confirming the animal models, activity of MMP-9 is also elevated in the cerebrospinal fluid of patients with neuroinflammatory disease and it is expressed by invading leukocytes in MS lesions. MMP-9 was detected in the CSF by zymography, which is not a quantitative technique and is not specific for MMP-9 (Gijbels K et al. (1992) J Neuroimmunol. 41:29-34).

The CSF drains from the brain and spinal cord and lies within the blood-brain barrier. It is therefore not surprising that elevated levels of inflammatory mediators such as 92kDa gelatinase can be detected in the CSF. The central and peripheral nervous systems are immunologically privileged sites, with the blood-brain and blood-nerve barriers serving to restrict the passage of cells and proteins. Markers of a nervous system disease process would therefore not be expected to be detectable in quantity within the peripheral blood circulation.

Brief description of the invention

This invention is based on the surprising discovery that levels of MMP-9 are elevated in the blood of patients with MS and other neurological diseases and these levels have been found to rise in association with the development of clinical relapse. Rises in the level of a natural inhibitor of MMP-9, TIMP-1, are often associated with these rises (peaks) in MMP-9 levels. Levels of MMP-9 and TIMP-1 are therefore predictive of disease progression. These findings lead to a novel method for monitoring disease activity and clinical relapse in MS or other neurological diseases by measurement of

MMP-9 and/or TIMPs in samples of blood taken from patients.

Detailed description of the invention

According to a first aspect of the invention, there is provided a method for diagnosis or prediction of relapse or monitoring of disease activity in a patient with neurological disease comprising quantifying the amount of MMP-9, fragments thereof or allelic variants thereof, and/or a TIMP, fragments thereof or allelic variants thereof, present in a sample of blood extracted from the patient, and comparing that amount with a control.

According to a further aspect of the invention there is provided a method for diagnosis or prediction of relapse or monitoring of disease activity in a patient with neurological disease comprising quantifying the amount of MMP-9, fragments thereof or allelic variants thereof, present in a sample of blood extracted from the patient, and comparing that amount with a control.

According to a further aspect of the invention there is provided a method for diagnosis or prediction of relapse or monitoring of disease activity in a patient with neurological disease comprising quantifying the amount of a TIMP, fragments thereof or allelic variants thereof, present in a sample of blood extracted from the patient, and comparing that amount with a control.

The level of MMP-9 or TIMP detected is to be compared to a control (also termed standard) which may be guaged from the average levels of MMP-9 and/or TIMP in the blood of normal individuals, patients at certain stages of specific neurological diseases or disorders, or from the individual patient themselves taken previously outside a relapse period and when the levels are at their basal level. For monitoring disease progression it is preferred to take a series of blood samples at periodic intervals so as to establish the basal MMP-9 or TIMP levels, thus enabling the clinician monitoring the patient to accurately detect rises in MMP-9 and/or TIMP levels which may be predictive of disease progression and relapse. Having determined the basal/control concentration of the

MMP-9 or TIMP in the blood, a comparison of this value with that present in the blood of a patient under study can be made.

The inventors have detected raised MMP-9 in the blood of patients suffering from various neurological diseases including: cerebrovascular disease, Parkinson's disease, motor neuron disease, epilepsy, encephalopathy, complications of diabetes, Miller-Fisher syndrome, tumours of the nervous system and multiple sclerosis. A number of these diseases involve demyelination. In addition, peaks in MMP-9 were often found to be associated with a peak in TIMP-1 levels. It is predicted that this invention can also be used to assess the status of patients suffering from other types of neurological diseases such as stroke and myasthenia gravis.

The method of the invention can be used to detect or monitor progression of any neurological disease in which MMP-9 is involved, but is particularly suitable for detecting and monitoring demyelinating diseases such as multiple sclerosis.

The invention is directed to a method for the assessment of the condition of a patient suspected, or known to have, multiple sclerosis or any neurological disorder in which MMP-9 levels in the blood give a measure of disease activity. Assessment of disease activity permits the employment of various preventive or remedial treatment regimes best suited for the condition.

The method comprises quantification of MMP-9 and/or a TIMP (particularly TIMP-1) and/or an MMP-9/TIMP complex, including allelic variants, active or inactive fragments or proteinaceous degradation productsof these molecules, in samples of blood extracted from the patient, wherein the changes in amount of any form of MMP-9 or TIMP indicates, or predicts, a change in disease activity of the patient.

In a preferred embodiment, the method comprises quantification of MMP-9, including allelic variants or any active or inactive fragments of the molecule, including proteinaceous degradation products, in samples of blood extracted from the patient,

wherein the changes in amount of any form of MMP-9 indicates, or predicts, a change in disease activity of the patient.

Allelic variants of MMP-9 are those which possess, in increasing order of preference, at least 90%, 95%, 97%, 98% or 99% amino acid identity to that of the published sequence (SM Wilhelm et al (1989) J Biol. Chem. 264(29):17213, erratum published in J Biol Chem 265(36):22750). Activity refers to the ability of the enzyme to cleave its various protein substrates. Thus the methods of the invention can rely on detection of enzymatically active MMP-9 or the inactive zymogen precursor of MMP-9. Detection of MMP-9 need also not be to the whole protein, as cleaved peptides of MMP-9 present in the blood will also be detectable.

Allelic variants of TIMPs are those which possess, in increasing order of preference, at least 90%, 95%, 97%, 98% or 99% amino acid identity to that of the published sequence of the TIMP in question. The preferred TIMPs are TIMP-1 and TIMP-3 whose amino acid sequences are present on the EMBL database under accession numbers: X03124 and X76227 respectively. Activity refers to the ability of the TIMP to inhibit the ability of metalloproteinases, such as MMP-9, to cleave their various protein substrates. Detection of the TIMP in question need not be to the whole protein, as cleaved peptides of the TIMP present in the blood will also be detectable. The methods of the invention can also rely on detection of MMP-9/TIMP complex.

MMP-9 and TIMP-1 blood levels have been found to rise around the time of relapse. A rise in MMP-9 and/or TIMP-1 prior to the clinical rise in EDSS for the patient should enable prediction and monitoring of a clinical relapse of MS. EDSS, the expanded disability status score (JF Kurtze (1965) Neurology. 15:654-661) is a universally recognised method of scoring disability in MS patients.

The invention places importance on the role of MMP-9 in the etiology of neuroinflammatory diseases such as multiple sclerosis. The MMP-9 referred to is also known as 92kDa gelatinase or type IV collagenase, gelatinase-B and EC 3.4.24.35. The

human MMP-9 was cloned and sequenced by SM Wilhelm et al and reported in SM Wilhelm et al. (1989) J Biol. Chem. 264:17213. The human MMP-9 sequence is present on the EMBL database (accession number:J05070) and Swissprot database (accession number:P14780). The precursor form of MMP-9 is a glycoprotein of 707 amino acids with an observed molecular weight of Mr 92,000 and an NH_2 terminal sequence of APRQRQ. On activation by proteolysis or organomercurial compounds such as p-aminophenyl mercuric acid, MMP-9 is processed to a smaller form of about Mr 86,000 with an NH_2 terminal sequence of FQTFEG. Other processing variants of this molecule have been observed.

The proenzyme has five domains: an amino terminal pro-domain and a catalytic domain shared by all members of the MMP family known so far; a collagen-binding fibronectin-like domain which is also present in 72kDa gelatinase; a carboxy terminal hemopexin-like domain shared by all members of the family known so far except matrilysin; a 54 amino acid long proline-rich domain homologous to the $\alpha 2$ chain of type V collagen. The secreted form of MMP-9 is glycosylated.

The natural inhibitors of metalloproteinases are termed TIMPs (tissue inhibitors of metalloproteinases). To date, four TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been identified, cloned and sequenced. The amino acid sequences of these TIMPs are present in the EMBL database. TIMP-1 has accession number: X03124; TIMP-2 has accession number: J05593; TIMP-3 has accession number X76227; and TIMP-4 has accession number U76456.

The active form of MMP-9 (92kDa gelatinase) is often present *in vivo* as a complex with a TIMP which is released along with the enzyme. The molecular weight of active MMP-9 complex with TIMP-1 is approximately 120kDa, and complexed with TIMP-3 is approximately 117kDa. The TIMP complexed to MMP-9 in CSF has been previously shown to be primarily TIMP-3 using the reverse zymography method (Leco et al., J. Biol. Chem. 269(12):9352-9360, 1994). WO 97/09038 also discloses the identification of TIMP-3/gelatinase B complex formation in the CSF of patients with

acute multiple sclerosis.

The detection of TIMP/MMP-9 gelatinase complexes in the blood also forms part of the methods of this invention for the diagnosis, prediction or monitoring of neurological disease.

The amount of MMP-9 or TIMP present in a test sample of blood may be quantified, for example, by any assay or procedure which measures the amount of MMP-9 or the TIMP, or allelic variants thereof, including any active or inactive fragments of these molecules and comparing these amounts against standard control amounts of MMP-9 or TIMP. MMP-9 or TIMP can be detected by the use of specific antibodies that recognise any part of the MMP-9 or specific TIMP molecules, but which do not cross-react detectably with any other MMPs or TIMPs, and any formulation of assay incorporating the use of antibodies specific for MMP-9 or the specific TIMP. Both monoclonal and polyclonal antibodies are useful in the methods of the invention, and methods for the preparation of suitable antibodies are well known in the art. An enzyme linked immunosorbant assay (ELISA) is particularly suitable for measuring the MMP-9 or TIMP levels. The development and use of a suitable ELISA for detecting MMP-9 or TIMP levels in blood or fractions of blood can be performed by a person skilled in the art. Amersham Life Sciences sell an ELISA kit (code No. RPN 2611) for measuring TIMP-1 levels which is suitable for detecting TIMP-1 levels in blood according to this invention.

The invention also provides for a diagnostic kit for use in diagnosis or prediction of relapse or monitoring of disease activity in a patient with a neurological disease, particularly a neurological demyelinating disease such as multiple sclerosis.

More particularly, the invention provides for a diagnostic ex-vivo blood kit for use in diagnosis or prediction of relapse or monitoring of disease activity in a patient with demyelinating disease, said kit capable of being used for detecting levels of MMP-9, fragments thereof or allelic variants thereof in blood extracted from a patient.

The invention also provides for a diagnostic ex-vivo blood kit for use in diagnosis or prediction of relapse or monitoring of disease activity in a patient with demyelinating disease, said kit capable of being used for detecting levels of a TIMP, fragments thereof or allelic variants thereof in blood extracted from a patient.

A suitable diagnostic kit for detection of MMP-9 may utilise a sandwich ELISA. Such a kit may comprise a support surface, such as a plate, coated with an antibody specific for any part of MMP-9; purified human MMP-9 or a part thereof as a standard control,capable of being recognised by the specific antibody; monoclonal or polyclonal (non-human) anti-MMP-9 antibody labelled with a detection enzyme such as horseradish peroxidase and; a suitable detection substrate reagent such as tetramethylbenzidine.

Measurement of the amount of active MMP-9, or allelic variants, can also be made using any assay based on the cleavage of a substrate specific for the MMP-9 enzyme. MMP-9 can also be detected by extraction of the enzyme, or any part of the enzyme, from the test sample by a variety of techniques, including affinity purification using natural or recombinant substrates of the enzyme or specific antibodies. Quantification of the extracted MMP-9 can be performed by conventional techniques such as immunoassay, gel electrophoresis (incuding gelatin zymography) or assays based on the cleavage of substrates, including gelatin and quenched fluorescent peptide substrates such as in CG Knight et al. (1992) FEBS. 296(3): 263-266 .

A suitable diagnostic kit for detection of a TIMP may utilise a sandwich ELISA. Such a kit may comprise a support surface, such as a plate, coated with an antibody specific for any part of the TIMP; purified humanTIMP or parts thereof as a standard control, capable of being recognised by the specific antibody; monoclonal or polyclonal (non-human) anti-TIMP antibody labelled with a detection enzyme such as horseradish peroxidase and; a suitable detection substrate reagent such as tetramethylbenzidine.

A suitable ELISA kit for use in measuring levels of TIMP-1 in blood samples is that as

sold by Amersham Life Sciences (code No. RPN 2611).

Measurement of the amount of active TIMP, or allelic variants, can also be made using any assay based on the detection of inhibition of cleavage of a substrate specific for the MMP-9 enzyme. The TIMP can also be detected by extraction of the TIMP, or any part of the TIMP, from the test sample by a variety of techniques, including affinity purification using natural or recombinant substrates of the enzyme or specific antibodies. Quantification of the extracted TIMP can be performed by conventional techniques such as immunoassay, gel electrophoresis or assays based on the inhibition of cleavage of substrates specific for MMPs, including gelatin and quenched fluorescent peptide substrates such as in CG Knight et al. (1992) FEBS. 296(3): 263-266. The TIMP/MMP-9 complex can also be detected by for example, reverse zymography (Leco et al., J. Biol. Chem. 269(12):9352-9360, 1994).

The foregoing diagnostic, predictive and monitoring methods are particularly useful in patients with multiple sclerosis.

The invention also provides for a method for determining the optimum time for administration of treatment for multiple sclerosis comprising periodically extracting blood from a patient suffering from multiple sclerosis, testing the blood for the level of MMP-9, fragments or allelic variants thereof, and comparing that level with the basal level of MMP-9, fragments or allelic variants thereof, of that patient until a sufficiently high blood level of MMP-9, fragments or allelic variants thereof, for that patient has been reached at which time treatment should be effected.

Rosenberg et al. (WO 97/09038) measured gelatinase B and gelatinase B/TIMP complex in the CSF of multiple sclerosis patient suffering an acute exacerbation of the disease (raised EDSS score, MRI), before and after steroid treament. They observed elevated gelatinase B levels before treatment. Following steroid treatment, a drop in EDSS score, associated with a drop in gelatinase B level, was measured.

It is predicted that the MMP-9 level detectable in the blood of MS patients will also decrease following steroid treatment. The ability to accurately determine the change in levels of MMP-9 and/or TIMPs in the blood of patients suffering from neurological diseases, such as multiple sclerosis, will enable preventative and/or remedial therapy to be administered at the optimum time and for periods no longer than necessary, thus avoiding the severe toxicity associated with certain agents (such as high dose steroids) used in MS therapy.

The methods and kits of the invention will also be useful in monitoring the effects of drugs administered during periods of relapse.

As used herein, reference to blood includes whole blood as well as blood fractions such as serum.

The following experimental methods and data establish that MMP-9 is elevated in the blood of patients with multiple sclerosis and also in patients with other neurological diseases, when compared to blood donor controls (Example 1). An associated rise in levels of TIMP-1, a natural inhibitor of MMP-9, coinciding with raised MMP-9 levels has also been detected. The following experimental methods and data also establish that MMP-9 and TIMP-1 levels rise in association with a clinical relapse of MS in individual patients (Example 2), thus supporting the claims of the invention.

The following figures are referred to in the examples given:

Figure 1 MMP-9 levels (ng/ml) in the serum of patients with MS (n=27) or other neurological diseases (n=72) compared to the levels in sera from blood donor controls (n=38).

Figure 2 Longitudinal study of MMP-9 levels in sera from 8 patients with multiple sclerosis who experienced a clinical relapse during the course of the study. Key: ■ = MMP-9 (92kDa gelatinase) level; ● = EDSS.

Figure 3 Longitudinal study of a single patient suffering from multiple sclerosis over a 1 year period. MMP-9 (♦) and TIMP-1 (■) levels (ng/ml) are plotted against time (months). In this patient, two periods of relapse occurred, one approximately in May and the other approximately in December.

EXAMPLE 1: Measurement of MMP-9 levels in sera from patients with MS or other neurological disease and blood donor controls

ELISA assay for MMP-9

Preparation of recombinant human MMP-9

Standard molecular biology techniques were used to generate an expression vector containing the sequence for human MMP-9. Poly A+ RNA was prepared from human HT1080 cells (ECACC, Porton Down, UK) using a Fast Track mRNA Isolation Kit (Invitrogen Corporation, San Diego, CA, USA) and cDNA was synthesised using oligo-dT primers. The MMP-9 cDNA was amplified in segments by PCR, cloned into M13mp18 and pUC19 and sequenced. The fragments were then assembled into the complete cDNA and cloned into the expression vector pGW1HG as a KpnI / EcoRI fragment. pGW1HG is a vector described in Example 1 of WO-A-9109118. The sequence of the assembled gene was identical to the sequence published by SM Wilhelm et al. (1989) J Biol Chem. 264:17213-17221.

Production of recombinant cell line secreting MMP-9

The vector containing the MMP-9 cDNA was linearised with NotI and electroporated into Chinese Hamster Ovary (CHO) cells (ECACC, Porton Down, UK). Tissue culture media and supplements were obtained from Life Technologies Ltd, Paisley, Scotland. CHO cells were maintained in DMEM with 10% foetal calf serum and 2mM L-glutamine. For electroporation, CHO cells were suspended at 10^7 cells/ml in phosphate buffered saline at 4°C. 800μl of the suspension were placed into a sterile electroporation cuvette along with 40μl of NotI linearised cDNA at a concentration of 1mg/ml. After incubation for 10 minutes on ice, the cells were electroporated at 0.8kV

and 25mF, then left on ice for a further 20 minutes. The cells were plated out at 1000 cells/well into 5 96-well plates in growth medium. Recombinant cell lines were selected for expression of the xanthine-guanine phosphoribosyltransferase gene (GPT) carried on the pGW1HG vector by transfer to selection growth medium consisting of normal growth medium with the addition of 312.5 mg/l xanthine, 6.25 mg/l mycophenolic acid and hypoxanthine/thymidine (HT) supplement, adjusted to pH 7.2 with sterile HCl. Transfected cells were assayed for gelatinase expression by measuring the degradation of radioactively labelled gelatin (A Sellers et al. (1978) Biochem J. 171:493-496), and the highest producers were cloned out. For protein production, the selected clone was grown in CHO-S-SFM II. Medium was collected from the cells over several days, pooled and frozen at -20 °C until required for purification.

Purification of MMP-9

MMP-9 conditioned medium was loaded on to a gelatin Sepharose column at 4°C with 50mM Tris-HCl at pH7.4, 5mM calcium chloride, 0.05% Brij 35, 0.02% sodium azide (TCB). The column was washed with TCB buffer containing 0.15M sodium chloride and then eluted with TCB containing 1M sodium chloride and 10% (v/v) dimethyl sulphoxide.The eluate containing the purified enzyme was stored at -20°C.

The concentration of the purified protein was determined using the Bio-Rad Protein assay (Bio-Rad Laboratories Ltd, England) with bovine serum albumin as standard and was shown to be 0.12mg/ml. The enzyme produced by this method was shown to be 99% pure. The specific activity of the MMP-9 was determined by measuring the cleavage of a quenched fluorescent peptide substrate and was found to be 3230 mU/mg.

Production of a monoclonal antibody to MMP-9

A mouse monoclonal antibody (4H3) was produced to the human recombinant MMP-9 by standard techniques well known in the art. Briefly, this involved immunisation of a mouse with recombinant human MMP-9 to generate an immune response, removal of the spleen and dissociation of the splenocytes, followed by fusion with an immortal

myeloma cell line SP2/0 (ECACC, England). The resulting hybridomas were screened for production of antibody to MMP-9 by standard techniques involving the detection of antibody binding to the immunogen. Hybridomas producing antibodies to MMP-9 were cloned by limiting dilution and selection for strong recognition of the immunogen. Antibodies produced by these selected hybridoma lines were purified from the culture medium by affinity purification on protein A Sepharose. The monoclonal antibody used in the ELISA assay was selected based on its ability to act as a capture reagent for purified MMP-9 when bound to multiwell plates and for its specificity for MMP-9.

Production of a polyclonal antibody to MMP-9

A sheep polyclonal antibody was produced to MMP-9 by standard techniques well known in the art. Briefly, this involved immunisation of a sheep with purified, recombinant MMP-9 to generate an immune response. The immunoglobulin was purified from serum by protein A affinity chromatography and was then conjugated to horseradish peroxidase by R&D Systems Europe.

Measurement of MMP-9 in serum by ELISA

96-well microtitre plates (Nunc Immunosorb) were coated with a monoclonal antibody, 4H3, which is specific for MMP-9 at a concentration of 2.5μg/ml in carbonate/bicarbonate buffer pH9.6 (Sigma, Poole, England), 100μl/well for 16 hours at 4°C. The plates were washed three times with phosphate buffered saline without Ca^{2+} or Mg^{2+} (Life Technologies, Paisley, UK) (PBS). The remaining reactive sites were blocked by incubation for 1 hour at 4°C with phosphate buffered saline containing 1% bovine serum albumin (PBS-BSA), followed by three washes with phosphate buffered ssaline containing 0.1% Tween 20 (PBS-T). 100μl of test serum diluted 1:25 with phosphate buffered saline containing 1% bovine serum albumin + 0.1% Tween 20 (PBS-BSA-T) was added to duplicate wells. On each plate, a two-fold serial dilution of purified recombinant MMP-9 in PBS-BSA-T from 50ng/ml down to 0.78 ng/ml was set up to act as a standard. The plates were incubated for 2 hours at room temperature, then washed three times with PBS-T. Bound MMP-9 was detected by incubation of the plates with a horseradish peroxidase-conjugated sheep polyclonal antibody raised

against purified recombinant human MMP-9 at a concentration of 0.36μg/ml, 100ul/well in PBS-BSA-T. The plates were then washed three times with PBS-T and the horseradish peroxidase detected using 100μl/well of the substrate TM Blue (Universal Biologicals, London, UK). The reaction was allowed to proceed for 15 minutes at room temperature and was then stopped by the additon of 50μl/well of 1M hydrochloric acid. The optical density was read at 450nm using a Titertek plate reader (ICN Flow, UK) and concentration of MMP-9 in each test sample calculated from the standard curve using the Kineticalc program (Bio-Tek Instruments, UK).

The MMP-9 levels in 38 control serum samples from blood donors, 72 patients with a variety of neurological disorders including cerebrovascular disease, Parkinson's disease, motor neuron disease, epilepsy, encephalopathy, complications of diabetes, Miller-Fisher syndrome and tumours of the nervous system, and 27 from patients with multiple sclerosis were measured using the ELISA assay described above. The results shown in Figure 1 show that the level of MMP-9 is elevated in patients with MS or neurological disease compared to the levels found in controls.

In addition to blood serum, MMP-9 levels were also detectable in blood plasma.

Measurement of TIMP-1 in serum by ELISA

TIMP-1 levels were measured in serum samples extracted from the blood of normal individuals and multiple sclerosis patients using the TIMP-1 ELISA kit (Amersham Life Sciences, code No. RPN 2611) according to the manufacturer's instructions.

EXAMPLE 2: Measurement of MMP-9 and TIMP-1 by ELISA in a series of longitudinal serum samples taken from MS patients.

MMP-9 and TIMP-1 were measured in the serum of 40 patients with multiple sclerosis over a time period of up to one year using the ELISAs described in Example 1. Clinical assessments of each patient were made throughout the study and the EDSS for each patient recorded. During the course of the study, 8 patients were noted to have suffered

a relapse of the disease. The MMP-9 levels in these patients were plotted against the EDSS taken around the time of the relapses and are shown in Figure 2. The results show that in all eight patients there was a rise in MMP-9 levels associated with the rise in EDSS of the patients. The most severe relapse was associated with the greatest rise in the level of MMP-9. During the time-course study of the 40 patients, 32 separate elevations of MMP-9 (peaks) were noted outside of episodes of relapse or deterioration of progressive disease. Five of these were coincident with infections. The mean and median levels of MMP-9 were 491 and 378ng/ml respectively, with a range of 172-1085ng/ml. In a longitudinal study of 9 normal individuals, 8 elevations in MMP-9 were noted with mean and median levels of 271 and 231ng/ml respectively and a range of 112-666 ng/ml. However, within this control group, one elevation was coincident with an episode of cystitis, one with a broken leg and three with viral infection.

Sharp rises in TIMP-1 levels coincident with MMP-9 rises were observed in 5 of the 8 patients who suffered relapse. Suggesting that rises in TIMP-1 may be associated with rises in MMP-9 and disease activity.

Multiple sclerosis is characterised by clinical relapses each of which is associated with evidence of localised regions of inflammation (lesions) in the brain or spinal cord as detected by magnetic resonance imaging. Disease activity detected by MRI also proceeds outside of periods of clinical relapse, in fact for every clinical relapse there are about ten episodes of MRI activity (Thompson AJ et al, 1991, Ann. Neurol. 29:53-62). Whether a lesion is detectable as a clinical relapse depends on its size and location within the brain or spinal cord. It is therefore not surprising that changes in MMP-9 are detectable outside of periods of clinical relapse.

Figure 3 is a plot of the MMP-9 and TIMP-1 levels taken at monthly intervals in one of the patients who suffered relapse. Relapses occurred approximately in May and December. Sharp rises in both MMP-9 and TIMP-1 levels were detected at these relapse incidents. Peaks of MMP-9 and TIMP-1 were also observed outside of the relapse periods which may represent sub-clinical disease periods.

CLAIMS

1. A method for diagnosis or prediction of relapse or monitoring of disease activity in a patient with neurological disease comprising quantifying the amount of MMP-9, fragments thereof or allelic variants thereof, and/or a TIMP, fragments thereof or allelic variants thereof, present in a sample of blood extracted from the patient, and comparing that amount with a control.

2. A method for diagnosis or prediction of relapse or monitoring of disease activity in a patient with neurological disease comprising quantifying the amount of MMP-9, fragments thereof or allelic variants thereof, present in a sample of blood extracted from the patient, and comparing that amount with a control.

3. A method for diagnosis or prediction of relapse or monitoring of disease activity in a patient with neurological disease comprising quantifying the amount of a TIMP, fragments thereof or allelic variants thereof, present in a sample of blood extracted from the patient, and comparing that amount with a control.

4. A method as claimed in any one of claims 1 to 3 wherein the neurological disease is selected from the group consisting of: cerebrovascular disease, Parkinson's disease, motor neuron disease, epilepsy, encephalopathy, complications of diabetes, Miller-Fisher syndrome, tumours of the nervous system, multiple sclerosis, stroke and myasthenia gravis.

5. A method as claimed in any one of claims 1 to 4 wherein the neurological disease is a demyelinating disease.

6. A method as claimed in claim 5 wherein the demyelinating disease is multiple sclerosis.

7. A method as claimed in claim 1, claim 2, or any of claims 4 to 6 wherein the

amount of MMP-9, fragments thereof or allelic variants thereof are quantified using an immunoassay.

8. A method as claimed in claim 1, claim 3, or any of claims 4 to 6 wherein the amount of TIMP, fragments thereof or allelic variants thereof are quantified using an immunoassay.

9. A method as claimed in claim 7 or claim 8 wherein the immunoassay is an enzyme linked immunosorbent assay (ELISA).

10. A method as claimed in claim 1, claims 3 to 6, claim 8, claim 9, claim 17, or claim 19, or a kit as claimed in claim 15 wherein the TIMP is one selected from the group consisting of: TIMP-1, TIMP2, TIMP-3 or TIMP-4.

11. A method or kit as claimed in claim 10 wherein the TIMP is TIMP-1.

12. A diagnostic ex-vivo blood kit for use in diagnosis or prediction of relapse or monitoring of disease activity in a patient with neurological disease, said kit capable of being used for detecting levels of MMP-9, fragments thereof or allelic variants thereof in blood extracted from a patient.

13. A diagnostic ex-vivo blood kit for use in diagnosis or prediction of relapse or monitoring of disease activity in a patient with demyelinating neurological disease, said kit capable of being used for detecting levels of MMP-9, fragments thereof or allelic variants thereof in blood extracted from a patient.

14. A diagnostic kit as claimed in claim 13 wherein the demyelinating neurological disease is multiple sclerosis.

15. A diagnostic kit as claimed in any one of claims 12 to 14 comprising: a support surface coated with an antibody specific for any part of MMP-9; purified human MMP-9

or a part thereof as a standard control, capable of being recognised by the specific antibody; monoclonal or polyclonal (non-human) anti-MMP-9 antibody labelled with a detection enzyme and a suitable detection substrate reagent.

16. A diagnostic kit as claimed in any one of claims 12 to 14 comprising: a support surface coated with an antibody specific for any part of a TIMP, a purified human TIMP or a part thereof as a standard control, capable of being recognised by the specific antibody; monoclonal or polyclonal (non-human) anti-TIMP specific antibody labelled with a detection enzyme and a suitable detection substrate reagent.

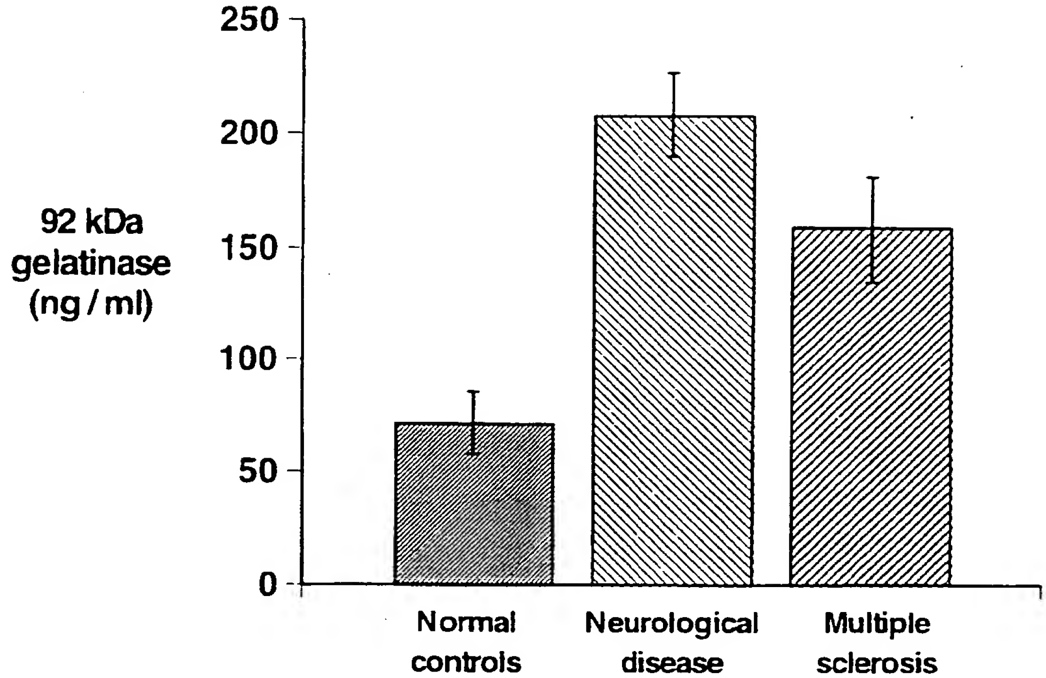
17. A method of monitoring disease progression in a patient suffering from multiple sclerosis comprising extracting blood from the patient, testing the blood for the level of MMP-9, fragments or allelic variants thereof, and/or a TIMP, fragments or allelic variants thereof, present in the blood and comparing that/those level(s) with previous levels of MMP-9, and/or a TIMP, fragments or allelic variants thereof in the blood from the same patient.

18. A method of monitoring disease progression in a patient suffering from multiple sclerosis comprising extracting blood from the patient, testing the blood for the level of MMP-9, fragments or allelic variants thereof, present in the blood and comparing that level with previous levels of MMP-9, fragments or allelic variants thereof, in the blood from the same patient.

19. A method of monitoring disease progression in a patient suffering from multiple sclerosis comprising extracting blood from the patient, testing the blood for the level of a TIMP, fragments or allelic variants thereof, present in the blood and comparing that level with previous levels of that TIMP, fragments or allelic variants thereof, in the blood from the same patient.

20. A method for determining the optimum time for administration of treatment for multiple sclerosis comprising periodically extracting blood from a patient suffering from

multiple sclerosis, testing the blood for the level of MMP-9, fragments or allelic variants thereof, and comparing that level with the basal level of MMP-9, fragments or allelic variants thereof, of that patient until a sufficiently high blood level of MMP-9, fragments or allelic variants thereof, for that patient has been reached at which time treatment should be effected.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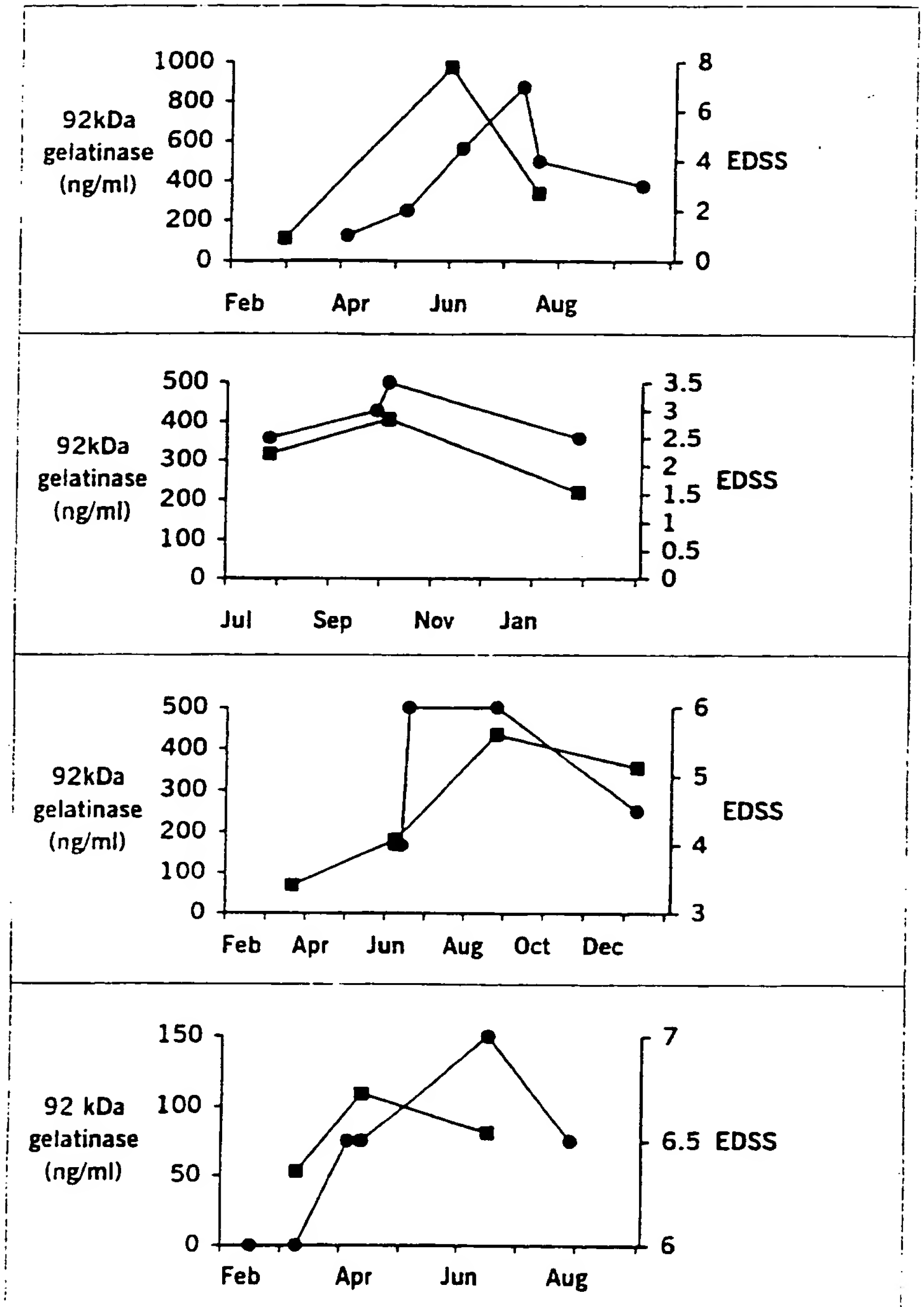
200
150
100
50
0
Normal controls
Neurological disease
Multiple sclerosis

Fig. 1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0
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6
4
2
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EDSS
Feb
Apr
Jun
Aug
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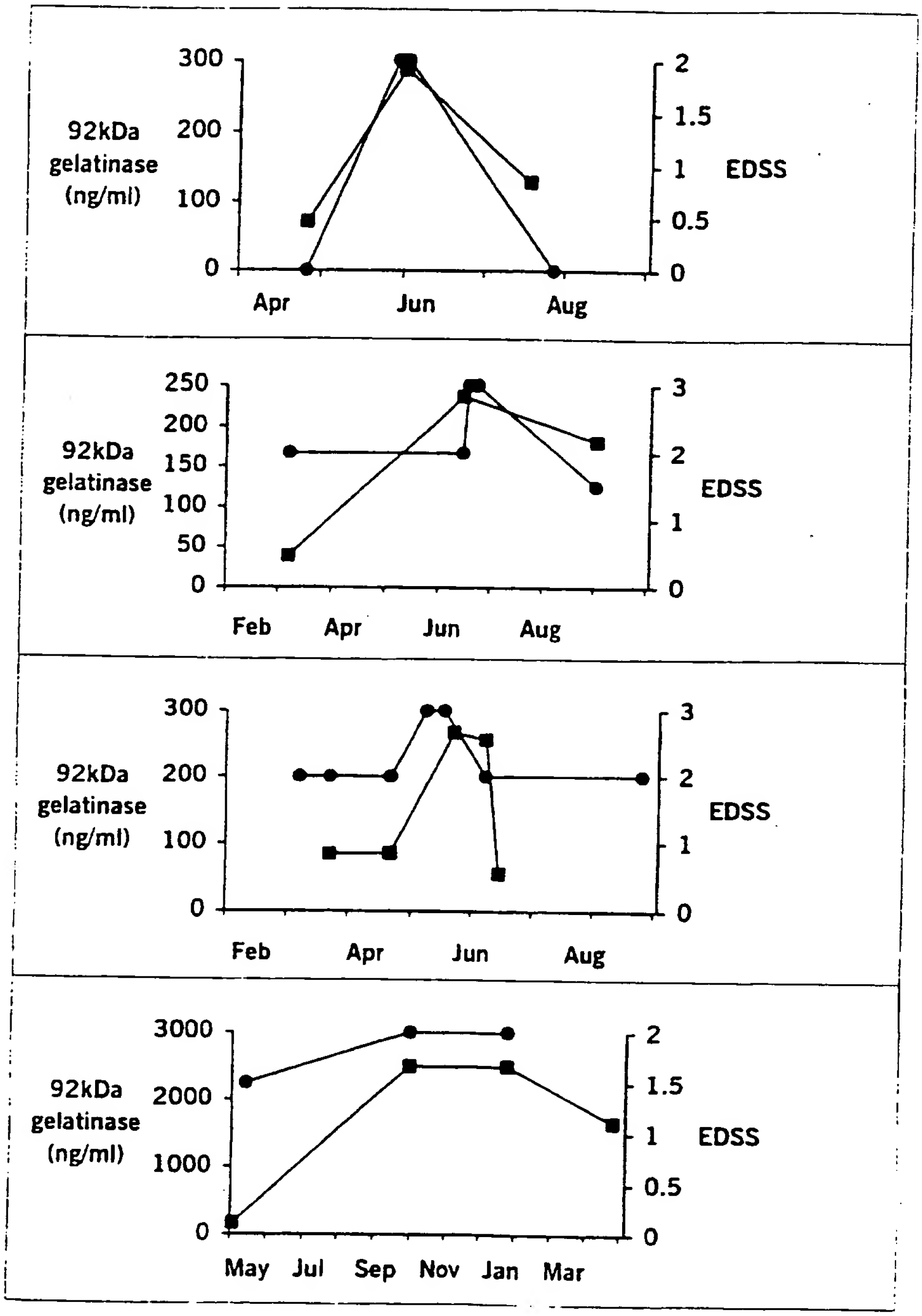
500
400
300
200
100
0
3.5
3
2.5
2
1.5
1
0.5
0
EDSS
Jul
Sep
Nov
Jan
92kDa gelatinase (ng/ml)
500
400
300
200
100
0
6
5
4
3
EDSS
Feb
Apr
Jun
Aug
Oct
Dec
92 kDa gelatinase (ng/ml)
150
100
50
0
7
6.5
6
EDSS
Feb
Apr
Jun
Aug

Fig. 2



92kDa gelatinase (ng/ml)
300
200
100
0
Apr
Jun
Aug
2
1.5
1
0.5
0
EDSS
92kDa gelatinase (ng/ml)
250
200
150
100
50
0
Feb
Apr
Jun
Aug
3
2
1
0
EDSS
92kDa gelatinase (ng/ml)
300
200
100
0
Feb
Apr
Jun
Aug
3
2
1
0
EDSS
92kDa gelatinase (ng/ml)
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1000
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Jul
Sep
Nov
Jan
Mar
2
1.5
1
0.5
0
EDSS

Fig. 2 contd.

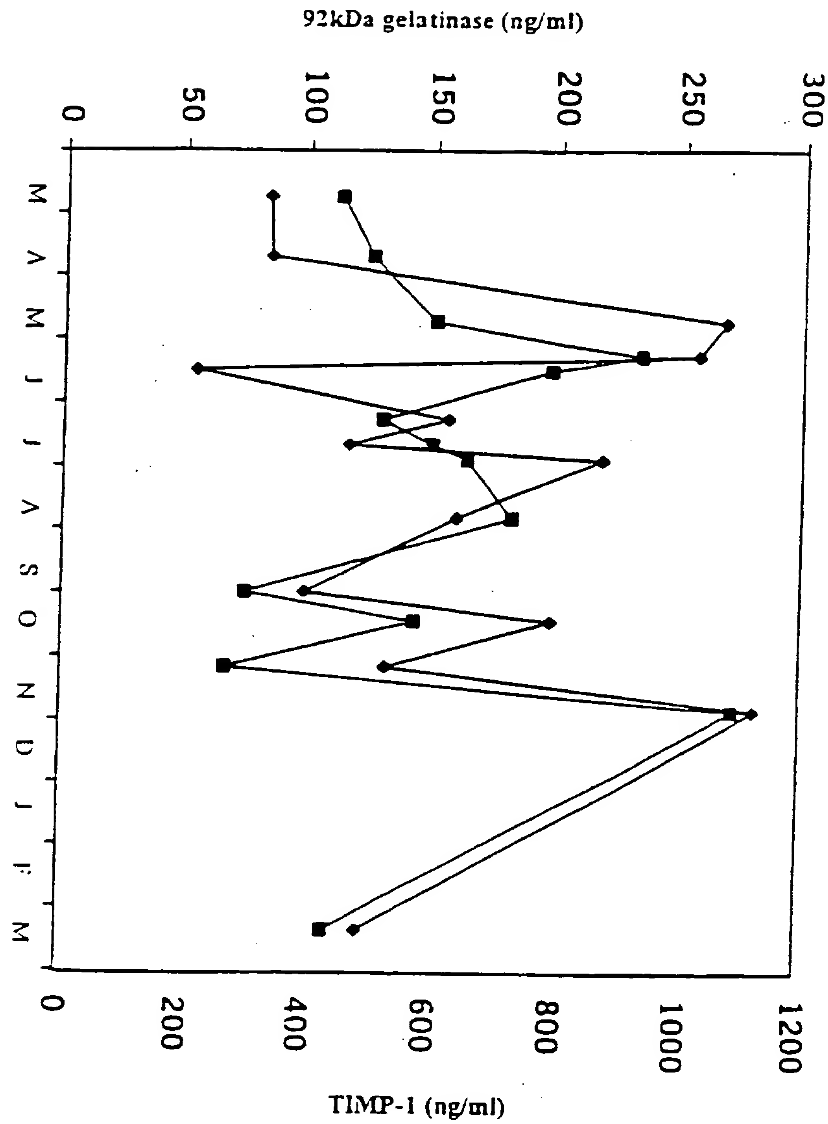
92kDa gelatinase (ng/ml)
0
50
100
150
200
250
300
M
A
M
J
J
A
S
O
N
D
J
F
M
0
200
400
600
800
1000
1200
TIMP-1 (ng/ml)

Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/00975

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/573 C12Q1/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 10208 A (MILES INC.) 11 May 1994 see the whole document ---	1-20
A	WO 90 11287 A (UNITED STATES OF AMERICA) 4 October 1990 see abstract; claims ---	1-20
A	WO 95 05478 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 23 February 1995 see abstract; claims ---	1-20
A	WO 95 31902 A (UNIVERSITY OF PENNSYLVANIA) 30 November 1995 see the whole document ---	1-20

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
14 August 1997	25 -08- 1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Griffith, G

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/00975

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 20447 A (THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK) 14 October 1993 see the whole document -----	1-20

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INTERNATIONAL SEARCH REPORT

.iormation on patent family members

Intern· ·nal Application No
PCT/GB 97/00975

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9410208 A	11-05-94	AU 5542794 A	24-05-94
		EP 0670849 A	13-09-95
WO 9011287 A	04-10-90	AU 634533 B	25-02-93
		AU 5359190 A	22-10-90
		CA 2046649 A	22-09-90
		EP 0464147 A	08-01-92
		JP 4504418 T	06-08-92
		US 5595885 A	21-01-97
WO 9505478 A	23-02-95	AU 7827794 A	14-03-95
WO 9531902 A	30-11-95	US 5641636 A	24-06-97
		AU 679858 B	10-07-97
		AU 2545695 A	18-12-95
		EP 0760601 A	12-03-97
WO 9320447 A	14-10-93	US 5324634 A	28-06-94
		AU 3940793 A	08-11-93